#39

1587-015-0 FWC I

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

TETSUJI SUDOH ET AL : EXAMINER: LEGUYADER

SERIAL NO: 08/192,800 :

FILED: FEBRUARY 7, 1994 : GROUP ART UNIT: 1805

FOR: PHYSIOLOGICALLY ACTIVE POLYPEPTIDE AND DNA :

DECLARATION UNDER 37 C.F.R. 1.132

HONORABLE COMMISSIONER OF PATENTS & TRADEMARKS
WASHINGTON, D.C. 20231

SIR:

Now comes _____, who declares and states that:

1. I am a graduate of _____ and received my _____ degree in the year _____.

2. I have been employed by _____ for ____ years as a _____ in the field of _____.

3. I have read the above-identified application, the Official Actions of April 21, 1994, October 6, 1993, January 22, 1993, July 30, 1991 and February 13, 1991, the references cited therein, the Amendments filed June 13, 1991, December 30, 1991, July 21, 1993, and the Preliminary Amendment filed February 7, 1994.

4. I understand that the present invention concerns:

a cDNA consisting essentially of a base sequence encoding a polypeptide having one of the following amino acid sequences:

(1) H-Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His-OH;

(2) H-Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His-OH;

(3) Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His;

(4) His Pro Leu Gly Ser Pro Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Leu Gln Glu Gln Arg Asn His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu Gln Thr Ser Leu Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg Glu Val Ala Thr Glu Gly Ile Arg Gly His Arg Lys Met Val Leu Tyr Thr Leu Arg Ala Pro Arg Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu; and

(5) Met Asp Pro Gln Thr Ala Pro Ser Arg Ala Leu Leu Leu Leu Leu Phe Leu His Leu Ala Phe Leu Gly Gly Arg Ser His Pro Leu Gly Ser Pro Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Led Gln Glu Gln Arg Asn His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu Gln Thr Ser Leu Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg Glu Val Ala Thr Glu Gly Ile Arg Gly His Arg

Lys Met Val Leu Tyr Thr Leu Arg Ala Pro Arg Ser Pro

Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met

Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val

Leu Arg Arg His;

a recombinant DNA sequence comprising a base sequence encoding one or more of polypeptides (1)-(5) above; and

a method of producing cDNA, comprising:

hybridizing a probe having a DNA sequence encoding a part of porcine brain natriuretic peptide to a human cDNA library;

selecting a positive clone; and

isolating the cDNA of the positive clone.

5. Neither the 70% degree of homology between human atrial natriuretic peptide (hANP) and porcine BNP (pBNP) taught by Sudoh et al (*Biochem. Biophys. Res. Comm.*, 155:726-732 and *Nature*, 332:78-80) nor the 50.6-65.7% degree of homology between hANP mRNA and pBNP mRNA taught by Maekawa et al is sufficiently high for one of ordinary skill to reasonably expect success in cloning and isolating the cDNA of one based on the sequence of the other.

6. Further, Table 1 of Oikawa et al teaches that the homology between hANP and other mammalian ANPs is only 52-60%. Thus, assuming that one of ordinary skill expects the same degree of homology between hBNP and other mammalian BNPs as is observed between hANP and other mammalian ANPs, Sudoh et al (*Biochem. Biophys. Res. Comm.*, 155:726-732 and *Nature*, 332:78-

80), Maekawa et al and Oikawa et al appear to indicate that the degree of homology is greater between pBNP and hANP than what one expects between pBNP and hBNP. As a result, one might expect a probe based on the pBNP gene to lead to cloning of a hANP gene, rather than a hBNP gene.

7. Sudoh et al (*Biochem. Biophys. Res. Comm.*, 159:1427-1434, attached hereto and incorporated herein by reference) disclose that human and porcine ANP's have 89.7% and 100% identical residues in the pro-form and α-form, respectively (page 1433, lines 1-3). However, the high homology between the pro- and α-forms of hANP and pANP would lead one to reasonable expect success in cloning and isolating hBNP cDNA using a 10-20 bp pBNP probe, which the present Inventors attempted but failed to successfully carry out.

8. Furthermore, the low homology (70.0%) between human prepro-BNP and porcine prepro-BNP (results determined by the present Inventors, disclosed by Sudoh et al [*Biochem. Biophys. Res. Comm.*, 159:1427-1434]) presents a sharp contrast to the more highly conserved mammalian ANP's, thus introducing an unexpected problem in cloning hBNP. This unexpected problem makes it surprising that hBNP cDNA could be cloned and isolated, given the level of ordinary skill and the knowledge in the art at the time of filing grandparent U.S. application Serial No. 07/486,827 (March 1, 1990).

9. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that

all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

10. Further declarant saith not.

_____
Signature

_____
Date

Vol. 159, No. 3, 1989
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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 1427-1434

CLONING AND SEQUENCE ANALYSIS OF cDNA ENCODING A PRECURSOR FOR HUMAN BRAIN NATRIURETIC PEPTIDE

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SUMMARY: Brain natriuretic peptide (BNP) is a novel diuretic-natriuretic and vasorelaxant peptide originally isolated from porcine brain. In contrast to mammalian atrial natriuretic peptide (ANP), immunological characterization suggests that mammalian BNPs show structural species differences. In order to determine the amino acid sequence of human BNP, we constructed a human cardiac atrium cDNA library and screened for clones hybridizing with porcine BNP cDNA. By sequence analysis of cDNA encoding a putative human BNP precursor, an amino acid sequence of human prepro-BNP of 134 residues has been deduced, in which a minimum bioactive unit highly homologous to porcine BNP-32 is present at the carboxy-terminus.

We have identified a new type of natriuretic peptide in porcine brain, i.e. brain natriuretic peptide of 26 residues (BNP-26) and its N-terminal extended form (BNP-32) (1,2). These peptides show remarkably high sequence homology to mammalian α-ANP, especially in the 17-amino acid ring formed by a disulfide linkage. In addition, they elicit pharmacological effects very similar to those of α-ANP, including diuretic-natriuretic, hypotensive and vasorelaxant activity. We have also identified pro-BNP of 106 residues (γ-BNP) as a major storage form in porcine cardiac atrium, although the atrial concentration of BNP is only 1-2% of that of ANP (3-5). These facts strongly suggest that a dual control mechanism through BNP and ANP generally exists in mammals for maintaining homeostatic balance of body fluid and electrolytes. In our preliminary study on BNP-like immunoreactivity in other mammals using a radioimmunoassay specific to porcine BNP-26, however, no crossreactivity was detected in human and rat tissue extracts, while slight and significant BNP-

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like immunoreactivity was observed in bovine and canine tissue extracts from heart and brain (unpublished observations). According to the immunological properties mentioned above, mammalian BNPs are suggested to show structural species differences in contrast to the highly conserved amino acid sequence of mammalian α-ANP. In order to help elucidate the as yet unclarified physiological functions of BNP, it is necessary to determine the amino acid sequence of human BNP. Fortunately, we have recently determined the amino acid sequence of a porcine BNP precursor by the cDNA cloning method and direct amino acid sequencing (5,6). By utilizing part of the cDNA of the porcine BNP precursor as a probe, we have cloned and determined the cDNA sequence encoding a putative human BNP precursor which we report here. The amino acid sequence of human BNP precursor is compared with that of porcine BNP precursor.

MATERIALS AND METHODS

cDNA library construction: Total RNA was extracted by the guanidine thiocyanate method from human atrial tissue obtained by autopsy from a 58-year old female. (This study was approved by the research ethics committee of Miyazaki Medical College.) Cardiac atrium was used as an mRNA source according to our previous study on porcine BNP. Poly(A)$^+$ RNA was purified from total RNA by oligo(dT)-cellulose chromatography (Pharmacia)(7). A cDNA library was constructed from 3 μg of poly(A)$^+$ RNA by the method of Gubler and Hoffman using a cDNA synthesis kit (Pharmacia)(8). After ligation of double strand DNA to EcoRI adaptor, a portion of cDNA was ligated to EcoRI-digested and dephosphorylated λgt10 DNA (Bethesda Research Laboratory), and then was used for in vitro packaging using Gigapack Gold (Stratagene). About 2.3×10^4 independent clones were obtained per nanogram of poly(A)$^+$ RNA.

cDNA library screening: A 120-base pair (bp) fragment corresponding to porcine prepro-BNP(92-131) was used as a probe for screening (6). This probe was prepared from XhoI and RsaI digestion of porcine BNP cDNA clone pBNP83 which harbored incomplete 320-bp long cDNA and was labeled with ^{32}P by the multipriming procedure (Amersham). The cDNA library was screened by plaque hybridization with the labeled probe in a hybridization solution (2×10^6 cpm/ml, 4 x SSC, 0.1% SDS, 5 x Denhardt's and 100 μg/ml salmon sperm DNA) for 16 h at 60°C. The filters were washed three times in 2 x SSC containing 0.1% SDS at 60°C each for 1 hr.

DNA sequencing: A clone (λhBNP57), which carried the longest cDNA insert, was used for the main sequencing. λhBNP57 was digested with EcoRI, and the cDNA insert was subcloned into plasmid BlueScript (Stratagene). Restriction fragments generated from the cDNA insert by digesting with relevant restriction endonucleases (SmaI, PstI and EcoRI) were re-subcloned into BlueScript and were sequenced by the dideoxy chain termination method using a sequencing kit (Toyobo)(9). Both strands of the cDNA clones were sequenced.

Northern blot analysis: Eight μg of poly(A)$^+$ RNA was denatured with 1 M glyoxal and 50% dimethylsulfoxide, electrophoresed on 1.5% agarose gel and transferred to Hybond-N nylon membrane (Amersham)(10,11). A 213-bp SmaI(26)/SmaI(239) fragment derived from λhBNP57 was labeled and used as a probe for hybridization.

RESULTS AND DISCUSSION

In the cloning and cDNA analysis of porcine BNP precursor, we employed a cDNA library prepared from poly(A)$^+$ RNA of porcine cardiac atrium (6), since tissue concentration of BNP immunoreactivity in porcine atrium was about 100 times higher than that in brain (4). On the analogy of BNP distribution in

porcine organs, we constructed a human atrial cDNA library for isolating clones harboring a human BNP precursor, although our antisera against porcine BNP-26 did not crossreact with human BNP and tissue concentration of human BNP was not yet measurable.

In the present study, about 4×10^5 recombinant phages in the constructed human cDNA library were screened by plaque hybridization with the 120-bp probe of porcine BNP. Under the hybridization conditions listed in Methods, clearly positive clones carrying a putative human BNP were found. Furthermore, these clones did not hybridize with a cDNA probe for human ANP of 581 bp under the same conditions. Under hybridization conditions of higher stringency, no positive clone was detected, suggesting that the positive clones thus obtained carried the human atrial cDNAs most homologous to porcine BNP precursor. Of 55 clones obtained by the screening, a clone designated λhBNP57, which harbored the longest cDNA insert of approximately 750 bp, was selected for further investigation. Clone λhBNP57 was sequenced by the dideoxy method according to the sequencing strategy shown in Fig. 1. The complete nucleotide sequence of 692 bp (excluding poly(A) tract and EcoRI adaptors) and the predicted amino acid sequence are shown in Fig. 2. Four other clones carrying shorter cDNA inserts were also sequenced by the same method. No nucleotide substitution was observed among the five clones sequenced in this study, though one substitution was noted in the case of porcine BNP precursor (6).

In the 692-bp nucleotide sequence of λhBNP57, a putative human BNP precursor was encoded as a single open reading frame of 134 amino acid residues, which started from an ATG initiation codon (nucleotide 1-3) and ended at a TAA termination codon (nucleotide 403-406) (Fig. 2). The hexanucleotide sequence AATAAA, which is known to precede poly(A) tract in many eukaryotic mRNAs, was located at nucleotide 572-577. Thus, this open reading frame was found to contain the whole structure of the putative human

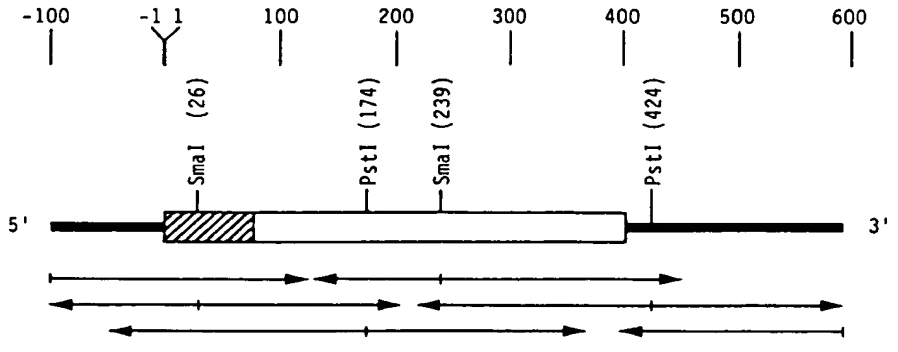

Figure 1. Strategy of sequencing the cDNA insert in clone λhBNP57. The restriction map displays only relevant restriction sites, which are identified by numbers indicating the 5'-terminal nucleotide generated by cleavages of the restriction enzymes. Each arrow shows the direction and extent of sequence determination. Putative human pro-BNP and the signal peptide are shown by open and hatched bars, respectively.

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                                                                5'·· ··· GGC  -97

-96  AGG CTG AGG GCA GGT GGG AAG CAA ACC CGG ACG CAT CGC AGC AGC AGC  -49

-48  AGC AGC AGC AGA AGC AGC AGC AGC AGC CTC CGC AGT CCC TCC AGA GAC   -1

  1  ATG GAT CCC CAG ACA GCA CCT TCC CGG GCG CTC CTG CTC CTG CTC TTC   48
  1  Met Asp Pro Gln Thr Ala Pro Ser Arg Ala Leu Leu Leu Leu Leu Phe   16
     -26

 49  TTG CAT CTG GCT TTC CTG GGA GGT CGT TCC CAC CCG CTG GGC AGC CCC   96
 17  Leu His Leu Ala Phe Leu Gly Gly Arg Ser His Pro Leu Gly Ser Pro   32
                                        -1 ▲ 1
 97  GGT TCA GCC TCG GAC TTG GAA ACG TCC GGG TTA CAG GAG CAG CGC AAC  144
 33  Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Leu Gln Glu Gln Arg Asn   48

145  CAT TTG CAG GGC AAA CTG TCG GAG CTG CAG GTG GAG CAG ACA TCC CTG  192
 49  His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu Gln Thr Ser Leu   64

193  GAG CCC CTC CAG GAG AGC CCC CGT CCC ACA GGT GTC TGG AAG TCC CGG  240
 65  Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg   80

241  GAG GTA GCC ACC GAG GGC ATC CGT GGG CAC CGC AAA ATG GTC CTC TAC  288
 81  Glu Val Ala Thr Glu Gly Ile Arg Gly His Arg Lys Met Val Leu Tyr   96

289  ACC CTG CGG GCA CCA CGA AGC CCC AAG ATG GTG CAA GGG TCT GGC TGC  336
 97  Thr Leu Arg Ala Pro Arg Ser Pro Lys Met Val Gln Gly Ser Gly Cys  112

337  TTT GGG AGG AAG ATG GAC CGG ATC AGC TCC TCC AGT GGC CTG GGC TGC  384
113  Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys  128

385  AAA GTG CTG AGG CGG CAT TAA GAG GAA GTC CTG GCT GCA GAC ACC TGC  432
129  Lys Val Leu Arg Arg His ***
                     108

433  TTC TGA TTC CAC AAG GGG CTT TTT CCT CAA CCC TGT GGC CGC CTT TGA  480

481  AGT GAC TCA TTT TTT TAA TGT ATT TAT GTA TTT ATT TGA TTG TTT TAT  528

529  ATA AGA TGG TTT CTT ACC TTT GAG CAC AAA ATT TCC ACG GTG AAA TAA  576

577  AGT CAA CAT TAT AAG CT(poly A)· ··· ·3'
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Figure 2. Nucleotide sequence of the cDNA insert in λ hBNP57 with predicted amino acid residues. Numbering of nucleotide residues begins with the first residue of ATG triplet encoding a putative initiating methionine, and nucleotides on the 5'-side of nucleotide 1 are indicated by negative numbers. Termination codon after BNP is marked with three consecutive asterisks, and hexanucleotide sequence preceding the polyadenylation site is underlined. The arrow indicates the most likely processing site for signal peptidase to give pro-BNP.

BNP precursor. The first 26 amino acid peptide, starting with the initiator methionine, was assumed to be a signal peptide, since this peptide consisted of a large number of hydrophobic amino acids and showed high sequence homology to the signal peptide of porcine BNP precursor (6,12). Nucleotide sequence 73-90 encoding Arg-Ser-His-Pro-Leu-Gly is identical to nucleotide 70-87 of porcine BNP cDNA sequence, in which the Ser-His bond is thought to be cleaved by a signal peptidase (5,6). Therefore, it is highly likely that the first processing of prepro-BNP takes place at the Ser-His bond to generate human pro-BNP of 108 residues in a manner similar to that of porcine BNP.

At the carboxy-terminal end of human prepro-BNP, there is a 32-amino acid peptide (encoded in nucleotide 307-402) highly homologous to porcine BNP-32. In this 32-amino acid sequence, 23 residues, including two cysteine residues

forming a 17-amino acid ring, are identical to porcine BNP-32 when aligned to show maximum matching. Thus, this carboxy-terminal peptide is deduced to be human BNP-32, which up to now has not been isolated. In the proteolytic processing pathway of porcine BNP in brain, pro-BNP is processed after a single arginine (1,2,6). Human BNP-32 is directly preceded by an arginine residue, just as is porcine BNP-32 within the precursor molecule. On the other hand, BNP-26 is also known to be present in porcine brain as a major form (3). In human brain, however, it is rather hard to presume the presence of BNP-26, since an arginine residue at the 27th position from the carboxy-terminus is replaced by a glutamine residue in the human pro-BNP sequence. Therefore, human BNP-32 is assumed to be a minimum endogenous form of human BNP generated from its precursor in the central nervous system and probably in the blood stream, although chemical identification of human BNP has yet to be performed. Furthermore, human BNP-32 is expected to elicit pharmacological effects similar to those of porcine BNP-32 and BNP-26, based on their structural homology.

Northern blot analysis showed that a single mRNA for human BNP precursor of about 900 bp was expressed in human atria (Fig. 3). This result also

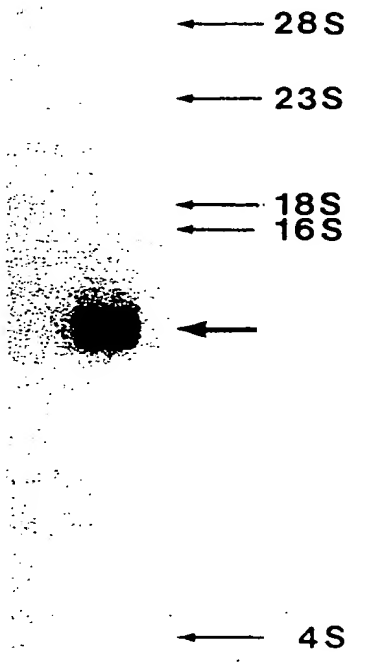

Figure 3. Northern blot analysis of messenger RNA of human atrium. SmaI(26)/SmaI(239) fragment derived from λhBNP57 was labeled and used as a probe. Hybridization-positive band is shown by the thick arrow. Positions of rat ribosomal RNAs (28S and 18S), bacterial ribosomal RNAs (23S and 16S) and bacterial transfer RNA (4S) are indicated by thin arrows.

indicated that human cardiac atrium contains a significant amount of BNP, as in the case of porcine atrium.

Nucleotide and amino acid sequences of human prepro-BNP are compared with those of porcine prepro-BNP in Fig. 4. When the 402-nucleotide sequence encoding human prepro-BNP is aligned to give maximum matching, it shows 70.0% homology to porcine BNP precursor (Fig. 4a). Nucleotide homology increases to 81.4% in the region corresponding to BNP-32 (nucleotide 307-402). On the other hand, this nucleotide sequence shows 57.2% homology to that for human prepro-ANP (13). At the amino acid sequence level, only 53.3% of the residues in human prepro-BNP are identical to those of porcine prepro-BNP, but 70.0% are identical when restricted to the carboxy-terminal 32 residues (Fig. 4b). Such a low homology in mammalian BNPs presents a sharp contrast

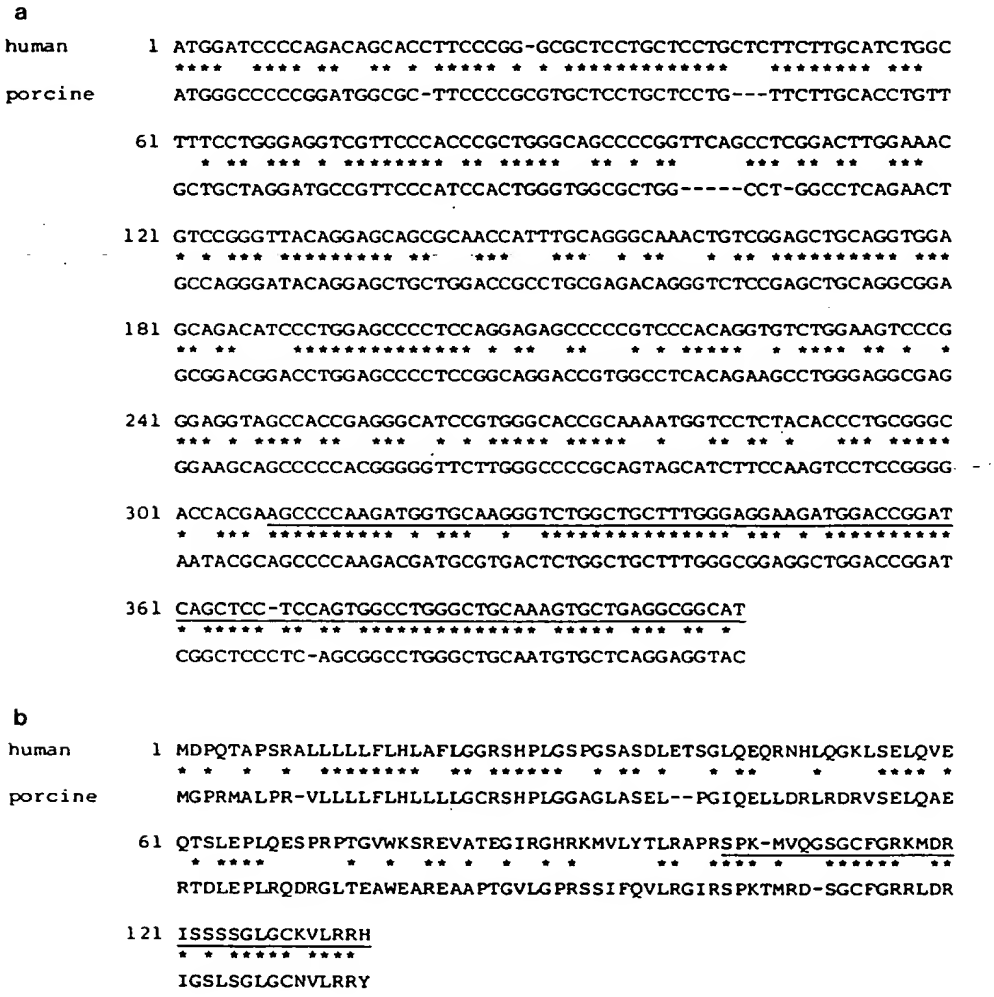

Figure 4. Nucleotide and amino acid sequence comparison of human BNP with porcine BNP. (a) nucleotide sequence. (b) amino acid sequence. Identical residues are shown with asterisks and the primary sequences corresponding to BNP-32 are underlined. Gaps (-) are introduced for maximal alignment. One letter notation for amino acid is used.

to the highly conserved amino acid sequences of mammalian ANPs, in which human and porcine ANPs have 89.7% and 100% identical residues in the pro-form and α-form, respectively (14). It should be noted that the 3'-untranslated region of human BNP cDNA has several repeptive ATTTA sequences, as is also observed in the porcine BNP cDNA sequence (6). Especially, nucleotide 497-529 in human BNP cDNA is highly AT-rich and shows 72.7% homology to porcine BNP cDNA. Since the repetition of ATTTA sequence is known to destabilize mRNA in the cell, mRNA encoding BNP may be transiently transcribed in response to stimuli and degraded in a short period (15,16). In contrast, mRNA encoding ANP does not contain the specific AT-rich sequence in the molecule (13,14). Although nucleotide and amino acid sequences of human and porcine BNPs do not exhibit extremely high homology, these two peptides share a well-conserved carboxy-terminal 32-amino acid sequence and a characteristic 3' AT-rich cDNA sequence. Therefore, the cDNA sequence determined in the present study is concluded to be a human BNP precursor. These structural data have demonstrated the relatively diverse sequences of mammalian BNPs in contrast to the highly conserved sequences of mammalian ANPs.

Structural elucidation of human BNP cDNA will facilitate identification of human BNP in brain, heart and blood. Furthermore, this will make it possible to understand how BNP functions in concert with ANP to maintain body fluid and electrolyte balance through neural and hormonal pathways in normal and diseased conditions.

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